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Chemotropic Responses of Retinal Growth Cones Mediated by Rapid Local Protein Synthesis and Degradation

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Summary

Growth cones contain mRNAs, translation machinery, and, as we report here, protein degradation machinery. We show that isolated retinal growth cones immediately lose their ability to turn in a chemotropic gradient of netrin-1 or Sema3A when translation is inhibited. Translation inhibition also prevents Sema3A-induced collapse, while LPA-induced collapse is not affected. Inhibition of proteasome function blocks responses to netrin-1 and LPA but does not affect Sema3A responses. We further demonstrate in isolated growth cones that netrin-1 and Sema3A activate translation initiation factors and stimulate a marked rise in protein synthesis within minutes, while netrin-1 and LPA elicit similar rises in ubiquitin-protein conjugates. These results suggest that guidance molecules steer axon growth by triggering rapid local changes in protein levels in growth cones.

Introduction

Axons navigate long distances to their targets using a succession of molecular cues expressed along the pathway (Tessier-Lavigne and Goodman, 1996). Netrin-1 and semaphorin 3A (Sema3A) are expressed in specific regions of the embryonic central nervous system where they act to attract or repel the growth of receptor-bearing axons (Colamarino and Tessier-Lavigne, 1995; Kobayashi et al., 1997). Netrin-1 at the midline, for example, attracts commissural axons and repels trochlear axons (Colamarino and Tessier-Lavigne, 1995; Kennedy et al., 1994) and, at the optic disc, directs retinal axons out of the eye (de la Torre et al., 1997; Deiner et al., 1997). Growth cones often travel long distances and have a high degree of autonomy from the cell body. Isolated growth cones can survive and grow in culture (Shaw and Bray, 1977) and maintain properties similar to intact neurons (Guthrie et al., 1989). Growth cone steering decisions are likely mediated locally because retinal axons navigate correctly in vivo after soma removal (Harris et al., 1987), yet the steps that lead from stimulus reception to steering are not understood.

Protein synthesis and degradation are highly regulated in the cell (Schubert et al., 2000) and act together in regulating cellular processes such as the cell cycle (Hershko, 1997; Pyronnet and Sonenberg, 2001) and synaptic plasticity, for example, long-term facilitation in *Aplysia* (Hegde et al., 1993; Martin et al., 1997). Vertebrate growth cones possess the machinery necessary for protein translation (Bassell et al., 1998; Tennyson, 1970) and can translate proteins locally (Crino and Eberwine, 1996; Davis et al., 1992). Developing dendrites harbor multiple different mRNAs (Crino and Eberwine, 1996) and, remarkably, inhibition of protein synthesis in hippocampal neurons blocks synaptic long-term depression (LTD) on the rapid timescale of 5 min (Huber et al., 2000). The majority of cellular proteins are degraded by the ubiquitin-proteasome pathway (Hershko and Ciechanover, 1998; Voges et al., 1999). Proteins targeted for destruction by the 26S proteasome complex are tagged with ubiquitin (Hershko and Ciechanover, 1998), an abundant highly conserved 76 amino acid protein found in all eukaryotic cells. The majority of cellular ubiquitin conjugates are targeted to the 26S proteasome, which degrades the substrates to small peptides (Hochstrasser, 1996). Genetic screens in *Drosophila* have identified mutations in the genes encoding a ubiquitin ligase (E3) and ubiquitin-specific protease enzymes such as *bendless* (Muralidhar and Thomas, 1993; Oh et al., 1994), *non-stop* (Martin et al., 1995; Poeck et al., 2001), and *ariadne* (Aguilera et al., 2000), which result in axon guidance defects. These findings suggest that both protein synthesis and degradation may be involved in growth cone guidance.

Since growing axons in vivo make rapid decisions, sometimes at large distances from their somas as they encounter new molecular territories along the pathway, we asked whether axon guidance is controlled by local translation and proteasome-mediated proteolysis. Here we show that Sema3A and netrin-1 stimulate rapid rises in the phosphorylation of translation initiation factor proteins and protein synthesis in isolated growth cones and that the chemotropic responses of retinal growth cones to these cues are blocked by the inhibition of protein synthesis. In addition, we show that netrin-1 and LPA simulate the production of ubiquitin-protein conjugates in growth cones and inhibition of proteasome-mediated proteolysis blocks chemotropic responses to these cues. Our results suggest that axons are guided by cues that trigger local rapid changes in protein levels within growth cones and that the responses elicited by different cues are mediated by distinct combinations of protein synthesis and/or degradation.

Results

Sema3A-Induced Chemotropic Responses Are Blocked by Protein Synthesis Inhibitors

To test the role of protein synthesis in growth cone guidance, we used two in vitro assays, collapse and chemotropic turning (Lohof et al., 1992; Luo et al., 1993). Repulsive factors such as Sema3A cause growth cones to lose their expanded motile form and collapse into a needle-shaped nonmotile tips (Luo et al., 1993) (Figures 1A and 1B). *Xenopus* retinal neurons collapse rapidly in response to Sema3A exhibiting peak collapse (75%–80%) after just 10 min (Campbell et al., 2001). Back-

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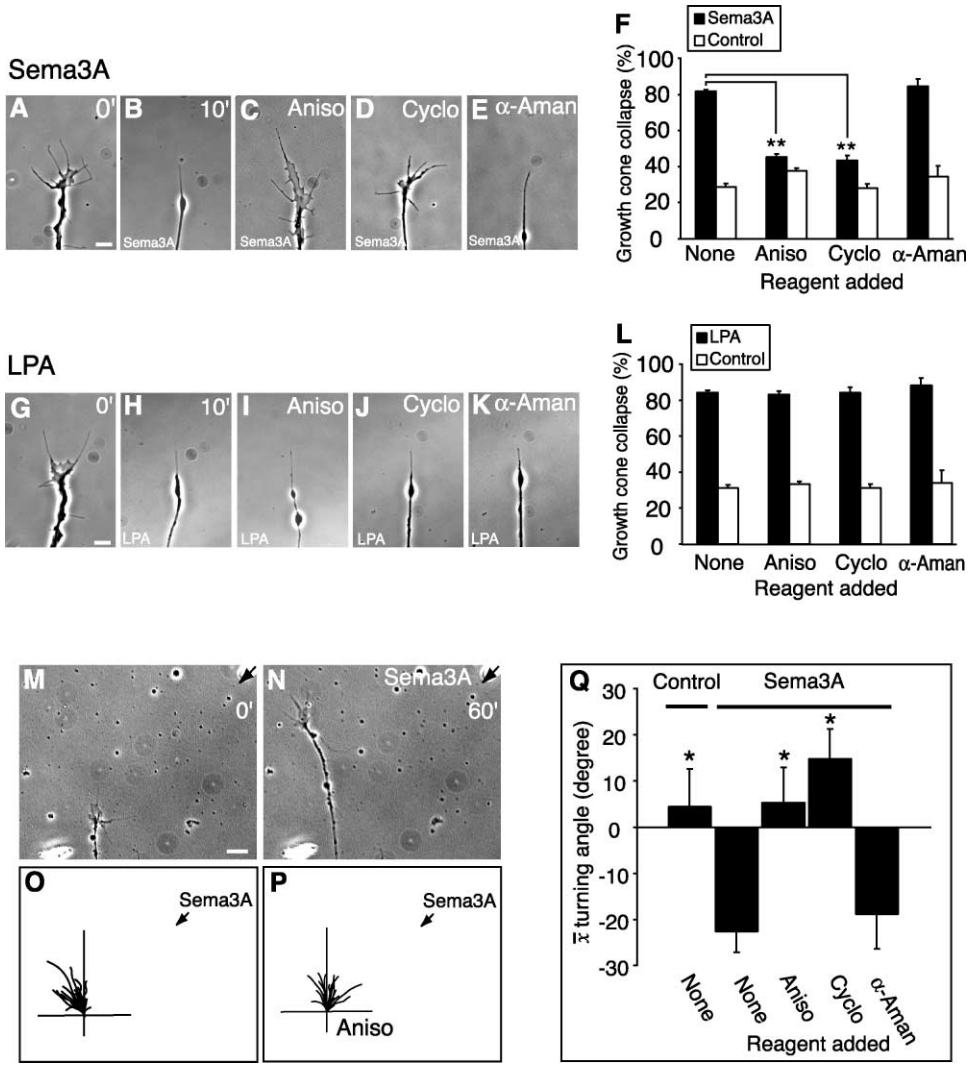

Figure 1. Protein Synthesis Inhibitors Prevent Sema3A-Induced Growth Cone Collapse and Chemotropic Turning

Sema3A induces collapse of stage 35/36 retinal growth cones in 10 min (A and B), which is inhibited by anisomycin (40 μM Aniso) and cycloheximide (25 μM Cyclo) but not α-amanitin (10 μg/ml α-Aman [C–F]). LPA induces retinal growth cone collapse in 10 min (G and H). LPA (1 μM) collapses growth cones in the presence of protein synthesis inhibitors and α-amanitin (G–L). Gradient of Sema3A causes repulsive turning of stage 32 growth cones over 1 hr (M–O). Sema3A-induced repulsive turning is significantly inhibited by protein synthesis inhibitors but not by α-Aman (P and Q). Protein synthesis inhibitor-treated conditions are significantly different from Sema3A (*$p < 0.05$) but not from controls ([Q], Kolmorogov-Smirnov test). Superimposed neurite trajectory plots in a Sema3A gradient in the absence (O) and presence of Aniso (P). The position of the growth cone at 0 min is at the intersection of the x and y axes, and positive turning angles are to the right and negative angles to the left. Pipette position is indicated by an arrow (M–P). **$p < 0.01$, t test; Mann-Whitney U test (F). Scale bars: 10 μm.

ground levels of collapse are typically 30%–40% in these neurons (Campbell et al., 2001). When the protein synthesis inhibitors anisomycin (Kang and Schuman, 1996) or cycloheximide (Obrig et al., 1971) are added to the cultures 30 s prior to the addition of Sema3A, collapse assayed at 10 min is almost completely blocked and growth cones maintain normal motile morphologies (Figures 1C, 1D, and 1F). By contrast, α-amanitin (Brehm et al., 1987), a transcription inhibitor, does not affect Sema3A-induced collapse (Figures 1E and 1F). Another collapsing reagent, L-α-Lysophosphatidic acid (LPA), which signals through G proteins (Saito, 1997), stimulates robust growth cone collapse, but protein synthesis inhibitors do not block this collapse (Figures 1G–1L). Therefore, protein synthesis inhibitors seem to selectively interfere with Sema3A-mediated collapse.

Does the repulsive turning of growth cones away from a source of Sema3A also require translation? A gradient of Sema3A was formed by pulsatile ejection from a micropipette positioned at an angle of 45° and 100 μm from a growth cone (Lohof et al., 1992) (Figures 1M and 1N), and the position and angle of the growth cone were measured after 1 hr. Like *Xenopus* spinal neurons (Song et al., 1998), stage 32 retinal growth cones typically exhibit negative turning angles (−20°) indicative of a strong repulsion to Sema3A (Campbell et al., 2001) (Figures 1M–1O and 1Q). By contrast, in the presence of protein synthesis inhibitors applied immediately prior to

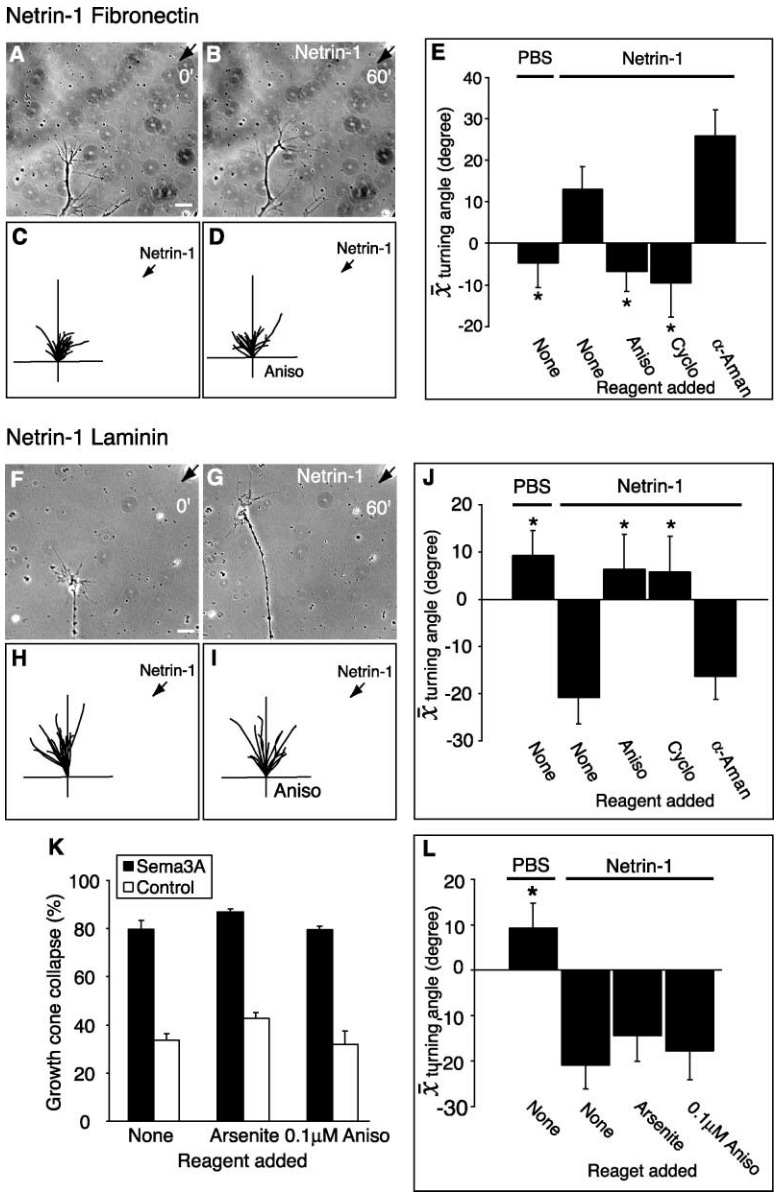

Figure 2. Netrin-1-Induced Attraction and Repulsion Are Blocked by Translation Inhibitors

Stage 24 retinal growth cones on 1 μg/ml fibronectin are attracted by a gradient of netrin-1 over 1 hr (A–C). Protein synthesis inhibitors but not α-amanitin inhibit netrin-1-induced growth cone attraction (D and E). Superimposed neurite trajectory plots in a netrin-1 gradient in the absence (C) and presence of Aniso (D). Stage 24 retinal growth cones grown on 10 μg/ml laminin are repelled by a gradient of netrin-1 over 1 hr (F–H). Protein synthesis inhibitors but not α-amanitin inhibit netrin-1-induced growth cone repulsion (I and J). Protein synthesis inhibitor-treated conditions are significantly different from netrin-1 (*$p < 0.05$) but not from PBS control ([E and J], Kolmogorov-Smirnov test). Sema3A-induced growth cone collapse (K) and netrin-1-induced repulsive turning (L) are unaffected by activation of the JNK/p38 MAP kinase stress pathway with 50 μM sodium arsenite (Arsenite) or 0.1 μM anisomycin (0.1 μM Aniso). *$p < 0.05$; Mann-Whitney U test (K); Kolmogorov-Smirnov test (L). Superimposed neurite trajectories with netrin-1 in the pipette and no reagent added to bathing medium (H) and in the presence of anisomycin (J). Arrows indicate position of pipette (A–D and F–I). Scale bars: 10 μm.

the start of the assay, repulsive turning is abolished and the growth cones advance with no directional bias (Figures 1P and 1Q). Neither the protein synthesis inhibitors nor α-amanitin had a significant effect on neurite length in the 1 hr turning assay period (mean neurite lengths: controls, 31 ± 4 μm; anisomycin, 34 ± 4 μm; cylohexamide, 38 ± 3 μm; α-amanitin, 37 ± 4 μm; $p > 0.1$, t test) showing that the inhibitors do not interfere with extension rate.

Attractive and Repulsive Turning in Response to Netrin-1 Is Prevented by Protein Synthesis Inhibitors

We next examined whether attractive turning responses of growth cones to guidance molecules require protein synthesis. Retinal growth cones from young (stage 24) eye explants are attracted toward netrin-1 (mean turning angle + 13°) (de la Torre et al., 1997; Höpker et al. 1999) (Figures 2A–2C and 2E). In the presence of protein synthesis inhibitors, however, growth cones show a neutral turning response demonstrating that netrin-1 attraction involves the synthesis of new proteins (Figures 2D and 2E). Again, α-amanitin had no effect on netrin-1-induced attraction (Figure 2E). When grown on laminin, retinal growth cones switch the direction of netrin-1-induced turning from attraction to repulsion (Höpker et al., 1999) and exhibit high negative turning angles (−20°) (Figures 2F–2H and 2J). This repulsive turning to netrin-1 is also blocked by protein synthesis inhibitors (Figures 2I and 2J).

In addition to blocking translation, protein synthesis inhibitors are known to activate the Jun terminal kinase (JNK)/p38 mitogen activated protein (MAP) kinase stress pathway (Cano et al., 1994). As a control for nontranslational effects of the protein synthesis inhibitors, we used sodium arsenite and an amount of anisomycin (0.1 μM) sufficient to activate the JNK/p38 MAP kinase pathway but not to inhibit translation (Liu et al., 1996). Unlike cycloheximide or 40 μM anisomycin, sodium arsenite

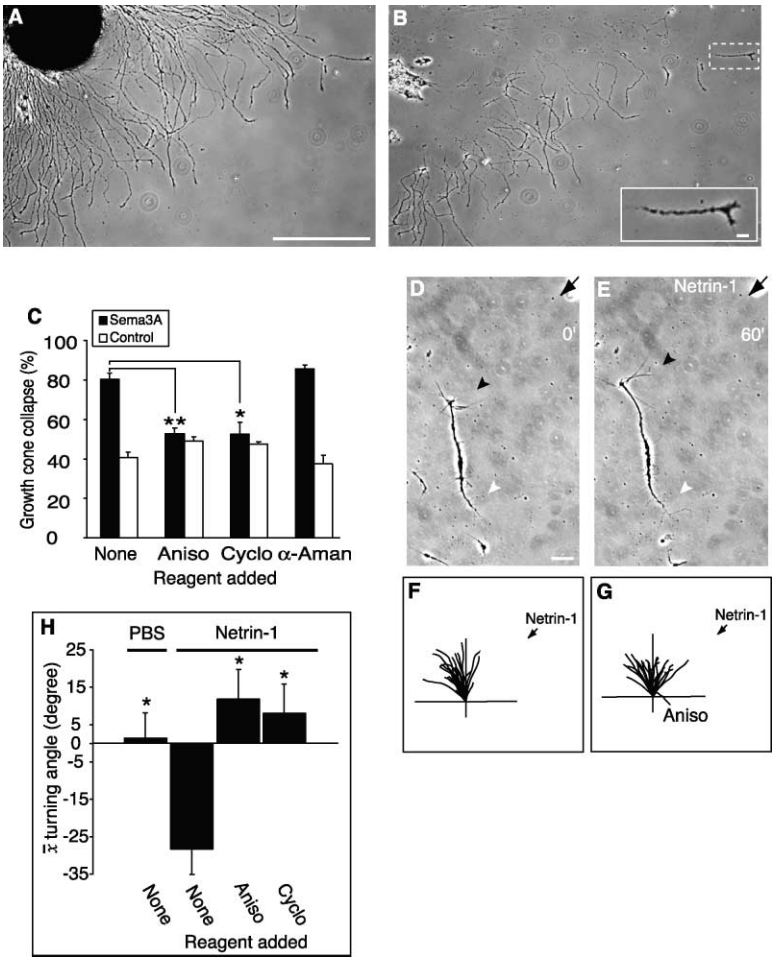

Figure 3. Isolated Growth Cones Exhibit Collapse and Chemotropic Turning, but Not in the Presence of Translation Inhibitors

Stage 24 retinal explants grown on 10 μg/ml laminin for 6 hr exhibit profuse neurite outgrowth (A). Somaless neurites remaining after explant removal (B). Inset, magnified view showing isolated growth cone morphology is maintained after explant removal. Protein synthesis inhibitors but not α-amanitin inhibit Sema3A-induced collapse of 24 hr stage 35/36 isolated growth cones (C). Somaless 6 hr stage 24 growth cones cultured on laminin are repelled by a gradient of netrin-1 over 1 hr (D–F). Isolated growth cone shows repulsive response to netrin-1 gradient (black arrowhead in [D] and [E]); white arrowhead in (D) indicates the cut end of the neurite. Protein synthesis inhibitors inhibit netrin-1-induced repulsion of isolated growth cones (G and H). Protein synthesis inhibitor-treated conditions are significantly different from netrin-1 only (*$p < 0.05$) but not from PBS control ([H], Kolmogorov-Smirnov test). Superimposed neurite trajectories with netrin-1 in the pipette and no reagent in the bathing medium (F) and in the presence of anisomycin (G). Arrow indicates position of pipette (D–G). *$p < 0.05$, **$p < 0.01$; Mann-Whitney U test (C). Scale bars: 100 μm (A), 10 μm (B and D).

or 0.1 μM anisomycin, which activate JNK in *Xenopus* retinal growth cones (data not shown), do not block Sema3A-induced collapse nor netrin-1-induced turning, showing that the JNK/p38 MAP kinase stress pathway is unlikely to be involved in regulating the chemotropic responses (Figures 2K and 2L).

Isolated Retinal Growth Cones Show Chemotropic Responses that Are Blocked by Protein Synthesis Inhibitors

Growth cones often begin to turn within 10 min of pipette placement, suggesting that local protein synthesis at the growth cone rather than remote synthesis at the cell body is of key importance. Because cell bodies are present in the cultures, the possibility remains that "turning" or "collapse" proteins are made in the soma and rapidly transported to the tip. Previous studies showed that growth cones without cell bodies continued to advance in culture (Shaw and Bray, 1977) and to navigate correctly in vivo (Harris et al., 1987). We used a similar approach to address the issue of local protein synthesis by removing the explant leaving behind severed somaless neurites attached to the substrate (Figures 3A and 3B). These neurites had normal growth cone morphologies (Figures 3B and 3D) prior to the addition of collapsing or turning agents and continued to exhibit normal collapse and turning responses and to grow for approximately 2 hr in culture (Figures 3C–3H). The turning and collapse responses were blocked by the addition of protein synthesis inhibitors demonstrating that the site of translation critical for growth cone chemotropic responses is local.

Translation Machinery Is Present in Retinal Growth Cones

These findings suggest that translational machinery in retinal growth cones is stimulated by guidance factors. To investigate the distribution of mRNA and ribosomes, we used an H20 anti-cap antibody that recognizes capped RNA (Bochnig et al., 1987) and an anti-ribosomal P0 antibody that recognizes a component of the 40S subunit (Muckenthaler et al., 1998). Growth cones showed strong positive staining with both antibodies that commonly extended into and along the filopodia (Figures 4A and 4B), demonstrating that key components of the translational machinery are present in growth cones.

Netrin-1 and Sema3A Rapidly Activate Translation Initiation Factor Proteins and Increase Protein Synthesis in Isolated Growth Cones

A key step in translation involves the release of a sequestered initiation factor, eIF-4E, from its binding protein, eIF-4EBP1. In its nonphosphorylated state, eIF-4EBP1

binds eIF-4E and represses translation. Phosphorylation of eIF-4EBP1 disrupts binding, releasing eIF-4E enabling it to bind the mRNA cap and activate translation (Gingras et al., 1999). Using an antibody that specifically recognizes phosphorylated eIF-4EBP1 (eIF-4EBP1-P), we asked whether we could detect a rise in eIF-4EBP1-P in growth cones in response to guidance factors. Just 5 min after the addition of Sema3A or netrin-1, but not LPA, the intensity of immunofluorescence in growth cones rose by at least 2-fold when compared to unstimulated controls (Figures 4C–4H). The phosphorylation of eIF-4E facilitates protein synthesis and increases its affinity for the mRNA cap (Minich et al., 1994). Similar intensity measurements using an antibody that specifically recognizes phosphorylated eIF-4E (eIF-4E-P) (Fraser et al., 1999) revealed that Sema3A and netrin-1 each induced a marked rise in eIF-4E-P in growth cones (Figures 4I–4N). The rise in fluorescence observed with eIF-4EBP1-P and eIF-4E-P was blocked by their respective phosphopeptides (Figures 4D and 4J). Thus, guidance factors inactivate the translation repressor eIF-4EBP1 and activate the translation initiation factor eIF-4E within minutes.

To measure changes in protein synthesis directly, ^{3}H-leucine was added to somaless cultures immediately before adding netrin-1, Sema3A, or LPA. After 10 min, the protein was precipitated with trichloroacetic acid (TCA) in situ, and the amount of radioactivity was measured. Low levels of incorporation were observed under control conditions (Figure 4O), and 2- to 4-fold increases in TCA-precipitated ^{3}H-leucine occurred in the presence of netrin-1 and Sema3A, but not with LPA. The increase was blocked by the protein synthesis inhibitors (Figures 4O and 4P). Thus, netrin-1 and Sema3A trigger rapid rises in local protein synthesis.

Netrin-1- and Sema3A-Induced Responses Differ in Their Requirement for PI-3 Kinase and Share a Common TOR-Dependent Pathway

Extracellular signals, such as insulin, commonly regulate protein synthesis through a phosphatidyl inositol-3 kinase (PI-3 kinase) and target of rapamycin (TOR)-dependent signaling pathway (Gingras et al., 1999). TOR directly phosphorylates eIF-4EBP1 leading to the release of the initiation factor, eIF-4E, and the initiation of translation. The PI-3 kinase pathway has previously been implicated in netrin-1-induced growth cone turning (Ming et al., 1999), leading us to suspect that it might be having this effect through its role on the control of translation. To begin to test the involvement of this pathway, we carried out growth cone collapse and turning assays in the presence of pharmacological inhibitors of PI-3 kinase (wortmannin and LY294002) and TOR (rapamycin). Rapamycin, but not wortmannin, inhibited Sema3A-induced growth cone collapse and turning (Figures 4Q and 4R). Moreover, rapamycin, but not wortmannin, inhibited Sema3A-induced ^{3}H-leucine incorporation (Figure 4T), whereas neither rapamycin nor wortmannin had an effect on LPA-induced growth cone collapse or ^{3}H-leucine incorporation (Figure 4T) (data not shown). Netrin-1-induced turning and ^{3}H-leucine incorporation were inhibited by wortmannin, LY294002, and rapamycin (Figures 4S and 4T) (data not shown). These results show that netrin-1 and Sema3A intracellular signaling pathways in retinal growth cones differ in their requirement for PI-3 kinase, as is the case for spinal neurons (Ming et al., 1999). However, they both act on the initiation of translation which is mediated by TOR.

Growth Cones Contain Components of the Ubiquitin-Proteasome System

Cellular processes that require tight regulation of protein levels commonly involve the degradation of proteins in parallel with synthesis. To determine whether proteasome-mediated proteolysis could play a role in chemotropic responses of growth cones, we first investigated the distribution of the proteasome and the highly related signalosome (Karniol and Chamovitz, 2000) in growth cones. Immunohistochemistry using antibodies to the 20S proteasome "core," the *Xenopus* proteasome α-4 subunit (Tokumoto et al., 1999) and the csn8 subunit of the COP9 signalosome revealed that all are present in growth cones and often extend into the fine filopodia (Figures 5A–5C). Ubiquitin and the ubiquitin-activating enzyme E1 were also detected in growth cones using antibodies (Figures 5D and 5E).

Proteasome Inhibitors Prevent Netrin-1-Induced Chemotropic Turning

We next examined whether proteasome-mediated proteolysis may play a role in chemotropic responses. In the presence of the cell permeant proteasome inhibitors N-Acetyl-Leu-Leu-NorLeu-Al (LnLL) (Pagano et al., 1995) and lactacystin (Fenteany et al., 1995), attractive turning toward a gradient of netrin-1 (Figures 5F–5J) was abolished. The mean turning angles were negative (-17° and -7°) but were not significantly different from PBS controls (Figures 5I and 5J). This result suggests that proteasome-mediated proteolysis is required for the attractive chemotropic response to netrin-1. Neurite lengths in 1 hr in the presence of proteasome inhibitors were unaffected (mean neurite lengths: controls, 24 $\pm$ 2 μm; LnLL, 27 $\pm$ 3 μm; lactacystin, 24 $\pm$ 3 μm; $p > 0.1$, t test). By contrast, a protease inhibitor cocktail containing the cell permeant non-proteasome protease inhibitors leupeptin, chymostatin, pepstatin A, and E64 does not affect netrin-1-induced attractive turning (Figure 5J). Like netrin-1-induced attractive turning, netrin-1-induced repulsive turning using laminin as a substrate is blocked by proteasome inhibitors but not by the protease inhibitor cocktail (Figures 5K–5O).

Chemotropic Responses Induced by LPA and BDNF, but Not Sema3A, Are Blocked by Proteasome Inhibitors

Since the Sema3A-induced collapse and turning are dependent on rapid translation, we examined whether these responses were similarly prevented by inhibitors of the proteasome. Both Sema3A-induced collapse and repulsive turning were unaffected by the application of proteasome inhibitors or a protease inhibitor cocktail (Figures 6A–6K). We next examined the effect proteasome inhibitors on LPA-induced collapse. Unlike the protein synthesis inhibitors, which had no effect on LPA-induced collapse, proteasome inhibitors completely blocked LPA-collapse (Figures 6L–6Q). The protease

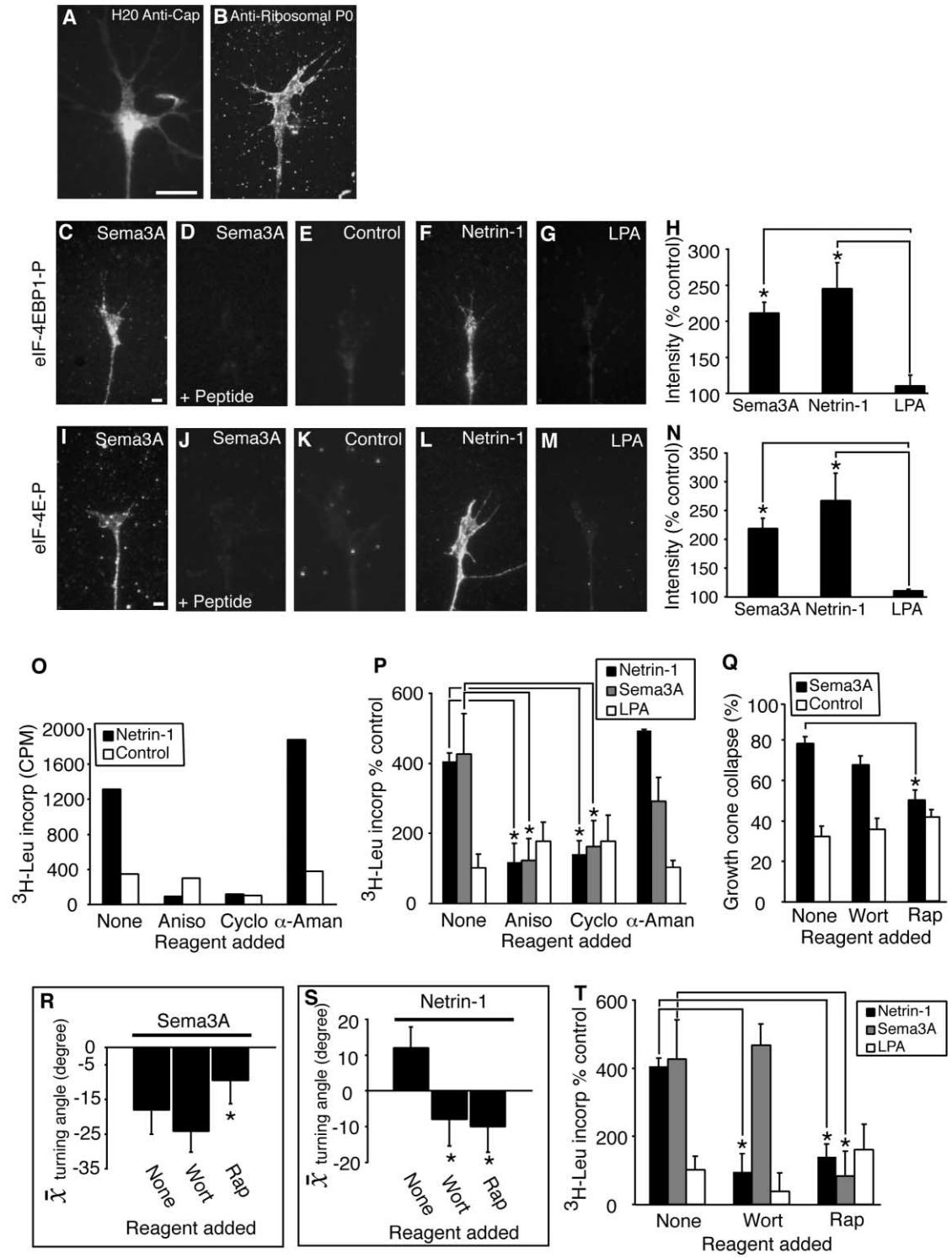

Figure 4. Local Translational Changes in Growth Cones Are Stimulated Directly by Guidance Molecules

Stage 24 retinal growth cones showing localization of mRNA ([A], H20 anti-cap antibody) and ribosomes ([B], anti-ribosomal protein P0 antibody). Phosphorylated forms (-P) of eIF-4EBP1 and eIF-4E in 24 hr stage 35/36 growth cones increase in response to 5 min treatment with Sema3A and netrin-1 compared with LPA or unstimulated growth cones (C–N). Vertical axes in (H) and (N) represent fluorescence intensity of treated growth cones as a percentage of untreated growth cones. Isolated growth cones in an example experiment incorporate low levels of TCA-precipitated ^{3}H-leucine in the absence of stimulation (counts per minute; CPM), which increase 3- to 4-fold in the presence of netrin-1 and is inhibited by protein synthesis inhibitors but not by α-amanitin (O). Netrin-1 and Sema3A increase the relative incorporation as a percentage of control TCA-precipitated ^{3}H-leucine in isolated growth cones, which is inhibited by protein synthesis inhibitors but not by α-amanitin (P). Rapamycin (Rap), but not wortmannin (Wort), inhibits Sema3A-induced collapse of stage 35/36 retinal growth cones (Q).

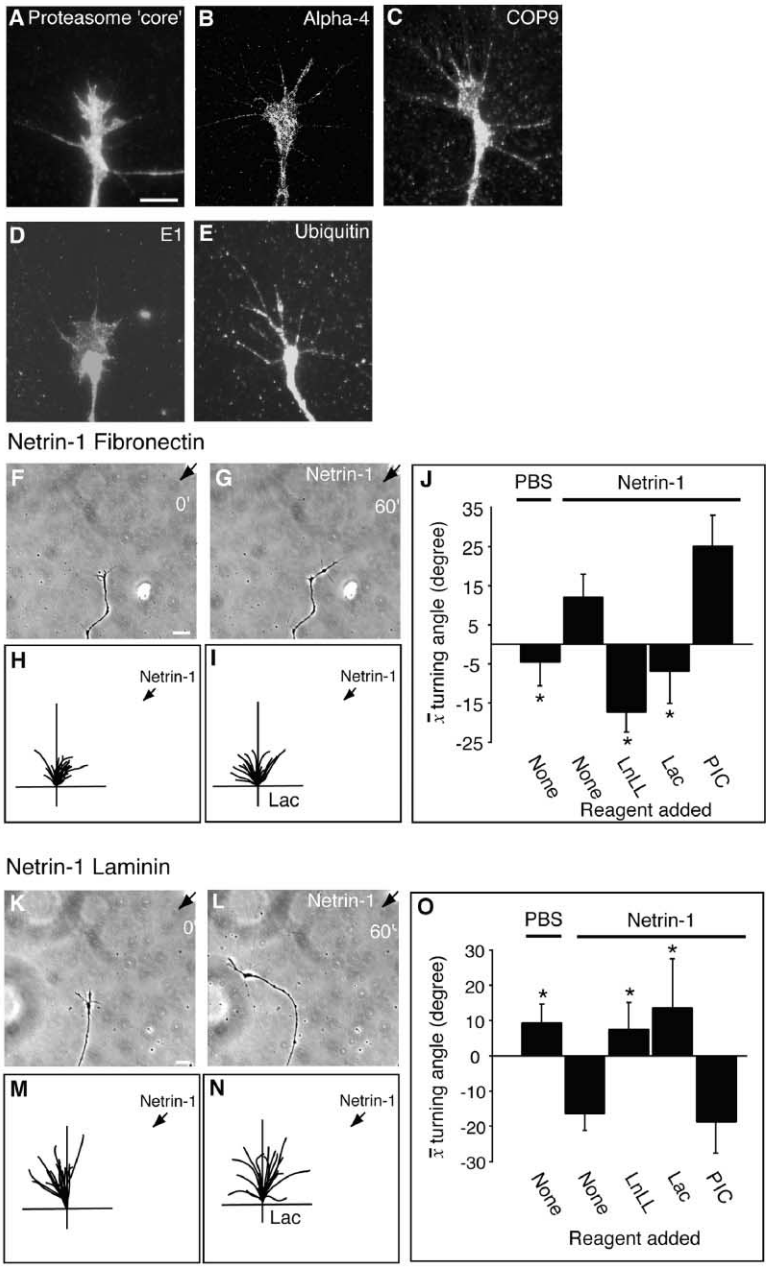

Figure 5. Retinal Growth Cones Contain Proteasome and Ubiquitination Pathway Components

Stage 24 retinal growth cones showing localization of proteasome ([A], 20S proteasome "core," and [B], anti-α-4 subunit antibodies), signalosome ([C], anti-COP9 [csn8] antibody) and ubiquitin system ([D], anti-ubiquitin, and [E], anti-ubiquitin activating enzyme E1 antibodies). Stage 24 retinal growth cones on 1 μg/ml fibronectin are attracted by a gradient of netrin-1 over 1 hr (F–H). Netrin-1-induced attractive turning is inhibited by the proteasome inhibitors LnLL (50 μM LnLL) and lactacystin (10 μM Lac) but not by the protease inhibitor cocktail (10 μg/ml PIC [I and J]). Stage 24 retinal growth cones grown on 10 μg/ml laminin are repelled by a gradient of netrin-1 over 1 hr (K–M). Proteasome inhibitors but not the protease inhibitor cocktail inhibit netrin-1-induced growth cone repulsion (N and O). Superimposed neurite trajectories with netrin-1 in the pipette and no reagent added to bathing medium (H and O) and in the presence of lactacystin (I and P). Proteasome inhibitor-treated conditions are significantly different from netrin-1 (*$p < 0.05$) but not from PBS control ([J and O], Kolmogorov-Smirnov test). Arrows indicate position of pipette (F–I and K–N). Scale bars, 10 μm.

inhibitor cocktail had no effect. Brain-derived neurotrophic factor (BDNF), a cAMP-dependent guidance cue (Song et al., 1997), induces attractive turning of retinal growth cones (Höpker et al., 1999), which is inhibited by proteasome inhibitors but not by the protease inhibitor cocktail (Figure 6S).

Chemotropic responses of growth cones to netrin-1 and Sema3A are dependent on cAMP and cGMP, respectively (Campbell et al., 2001; Ming et al., 1997; Song et al., 1998). Since the chemotropic responses of the cAMP-dependent guidance cues netrin-1 and BDNF are inhibited by proteasome inhibitors and those of Sema3A, which are cGMP-dependent, are not, we tested whether LPA-induced collapse was mediated by cAMP or cGMP. RpcAMPS, a membrane permeant antagonist of protein kinase A (PKA), inhibits LPA-induced collapse suggesting that cAMP mediates LPA-collapse of retinal growth cones (Figure 6R).

Rapamycin also reduces Sema3A-induced growth cone repulsive turning (R) and inhibits Sema3A-induced ^{3}H-leucine incorporation (T). Wortmannin and rapamycin both inhibit netrin-1-induced growth cone attraction (S) and netrin-1-induced ^{3}H-leucine incorporation (T). *$p < 0.05$; t test (H, N, O, and T); Mann-Whitney test (Q); Kolmogorov-Smirnov test (R and S). Scale bars: 10 μm.

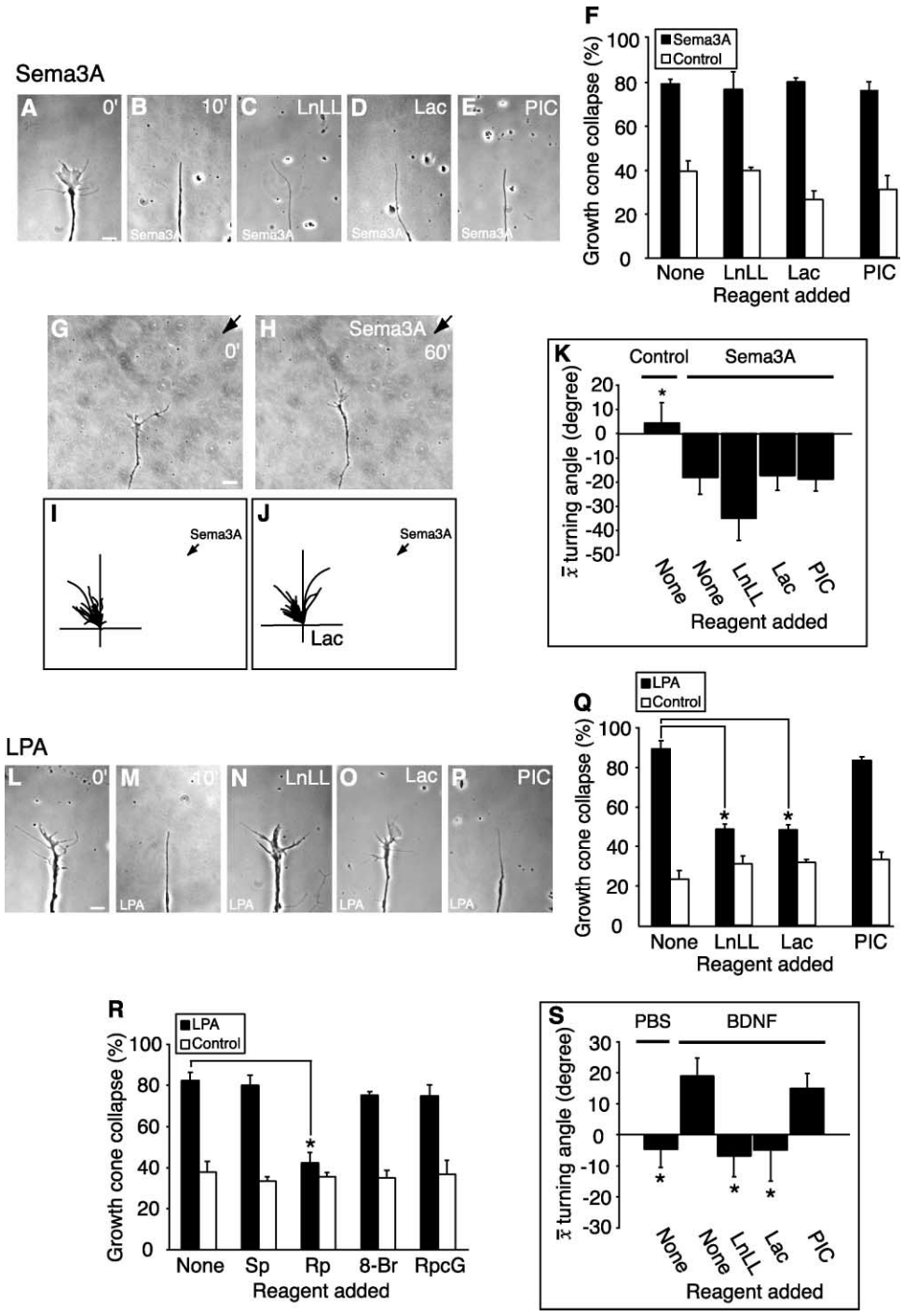

Figure 6. Sema3A-Induced Chemotropic Responses Are Unaffected by Proteasome Inhibitors

Sema3A induces collapse of stage 35/36 retinal growth cones in 10 min (A and B), which is unaffected by proteasome inhibitors or the protease inhibitor cocktail (C–F). A gradient of Sema3A causes repulsive turning of stage 32 growth cones over 1 hr (G–I). Repulsive turning is unaffected by proteasome inhibitors or the protease inhibitor cocktail (J and K) and is significantly different from controls (K; *$p < 0.5$ Kolmogorov-Smirnov test). Superimposed neurite trajectory plots with Sema3A in the pipette and no reagent added to the bathing medium (I) and in the presence of Lac (J). Pipette position is indicated by an arrow (G–J). LPA (1 μM) induces retinal growth cone collapse in 10 min (L and M). LPA-induced collapse is prevented in the presence of the proteasome inhibitors LnLL and lactacystin but not by the protease inhibitor cocktail (N–Q). LPA-induced growth cone collapse is inhibited by RpcAMPS (Rp), but not by SpcAMPS (Sp), 8-BrcGMP (8-Br), or RpcGMPS (RpcG [R]). BDNF causes attractive turning of stage 24 retinal growth cones over 1 hr (S). BDNF-induced attractive turning is significantly inhibited by proteasome inhibitors, but not by the protease inhibitor cocktail (S). Proteasome inhibitor-treated conditions are significantly different from BDNF (*$p < 0.05$), but not from controls ([S], Kolmorogov-Smirnov test). *$p < 0.05$; Mann-Whitney U test (Q and R). Scale bars: 10 μM.

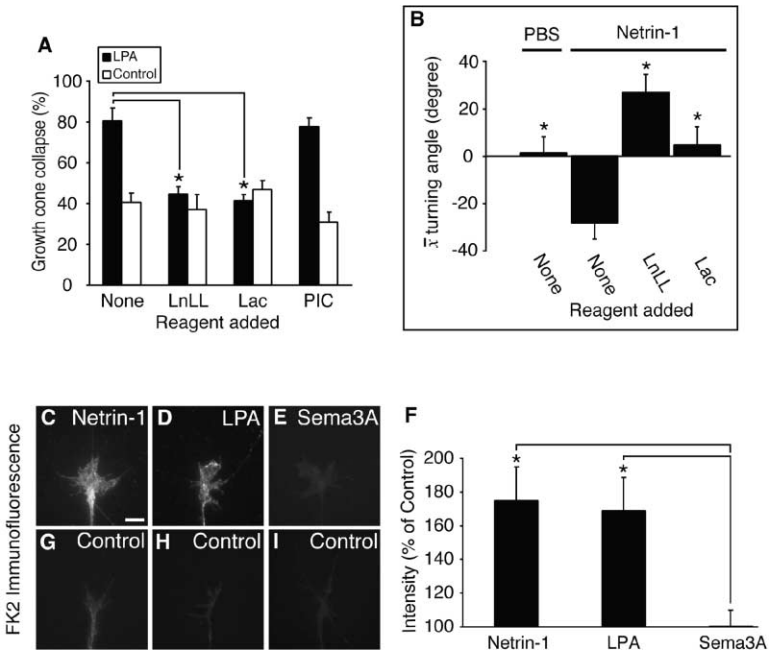

Figure 7. Isolated Growth Cones Exhibit Collapse and Chemotropic Turning but Not in the Presence of Proteasome Inhibitors

Protease inhibitors, but not the protease inhibitor cocktail, inhibit LPA-induced collapse of 24 hr stage 35/36 isolated growth cones (A). Somaless 6 hr stage 24 growth cones cultured on laminin are repelled by a gradient of netrin-1 over 1 hr (B). Proteasome inhibitor-treated conditions are significantly different from netrin-1 only (*$p < 0.05$) but not from PBS control ([B], Kolmogorov-Smirnov test). Ubiquitin-protein conjugates (FK2 immunofluorescence) in 24 hr stage 35/36 growth cones increase in response to 5 min treatment with netrin-1 and LPA compared with Sema3A (C–F) or unstimulated controls (G–I). Vertical axis in (F) represents fluorescence intensity of treated growth cones as a percentage of untreated growth cones. *$p < 0.05$; Mann-Whitney U test (A); t test (F). Scale bar: 10 μm.

Proteasome Inhibitors Block Chemotropic Responses in Isolated Growth Cones

To address the issue of local proteasome-mediated proteolysis directly, the cell bodies were again eliminated by removing the explant. Isolated growth cones exhibited normal collapse and turning responses to LPA and netrin-1. These responses were blocked by the addition of proteasome inhibitors (Figures 7A and 7B), indicating that local proteasome-mediated proteolysis events are critical for growth cone responses to netrin-1 and LPA.

Netrin-1 and LPA, but Not Sema3A, Elicit Rapid Rises in Ubiquitin-Protein Conjugates in Growth Cones

Proteins targeted for degradation by the proteasome are tagged with ubiquitin (Hershko and Ciechanover, 1998). Using an antibody that specifically recognizes ubiquitin-protein conjugates (FK2) (Everett, 2000; Fujimuro et al., 1994), we asked whether we could detect a rise in FK2 immunofluorescence in growth cones in response to chemotropic factors. 5 min after the addition of netrin-1 and LPA, but not Sema3A, the intensity of immunofluorescence in growth cones rose by approximately 1.8-fold when compared to unstimulated controls (Figures 7C–7I). Thus, netrin-1 and LPA stimulate rapid rises in ubiquitin-protein conjugates in growth cones.

Discussion

We have examined the role of local translation and proteasome-dependent proteolysis in mediating chemotropic responses of *Xenopus* retinal growth cones. We find that each of the three different cues investigated elicits responses through intracellular pathways that involve either translation or degradation or a combination of the two. Sema3A-induced responses require translation but not degradation; LPA-induced responses require degradation but not translation; while netrin-1-induced responses require both translation and degradation (summary diagram, Figure 8). Together, our data suggest that growth cone steering is likely to be mediated by rapid local changes in protein levels.

The rate of neurite extension continues normally in the presence of protein synthesis inhibitors during the duration of the 1 hr turning assays demonstrating that local protein synthesis is not required for growth. Previous studies have documented the supply of lipids and proteins (Hollenbeck and Bray, 1987; Popov et al., 1993; Zakharenko and Popov, 2000) from the cell body via vesicle transport to the growth cone. Presumably even in the isolated growth cones there is enough material en route in the severed neurite shaft to support insertion of new membrane required for growth for at least 1 hr. The current study reveals that the basal level of local protein synthesis in growth cones is low and only rises markedly when a guidance cue is added. Thus, it is likely that the protein synthesis occurring inside the growth cone is used primarily for navigation and not for extension.

In addition to their effects of inhibiting protein synthesis, anisomycin and cycloheximide are known to activate the JNK/p38 MAP kinase stress pathway (Cano et al., 1994). We have excluded a role for this pathway in the chemotropic responses to netrin-1 and Sema3A by using the JNK/p38 MAP kinase activator, sodium arsenite, which has no effect on the responses. Also, rapamycin inhibits netrin-1- and Sema3A-induced chemotropic responses and protein synthesis but does not activate the JNK/p38 MAP kinase stress pathway (Cuenda and Cohen, 1999; Ishizuka et al., 1997). Furthermore, we demonstrate independently of pharmacology that netrin-1 and Sema3A stimulate rapid rises in protein synthesis in isolated growth cones and trigger the phosphorylation of eIF-4E and eIF-4EBP1.

It is interesting to consider the speed and amount of

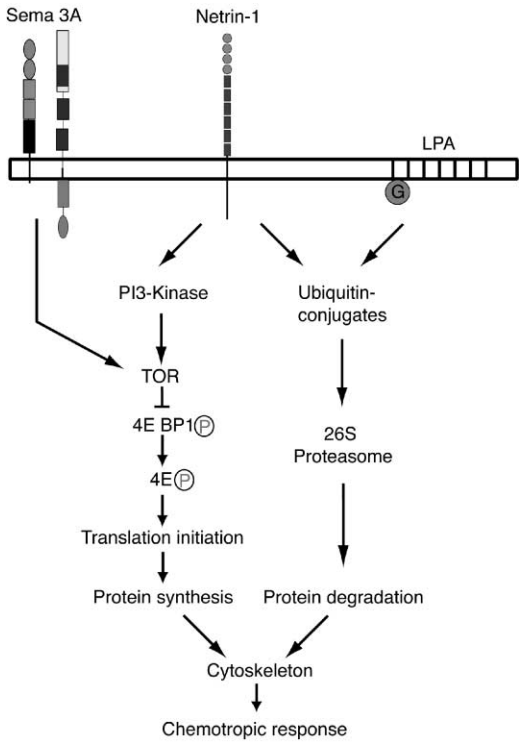

Figure 8. Summary of Distinct Translation/Degradation Pathways that Mediate Netrin-1, Sema3A, and LPA Chemotropic Responses

Netrin-1-induced chemotropic responses in growth cones require protein synthesis and degradation via a PI-3 kinase- and TOR-dependent pathway. Sema3A induces chemotropic responses via protein synthesis, but not degradation, in a PI-3 kinase-independent TOR-dependent fashion. LPA-induced collapse is independent of protein synthesis, PI-3 kinase, and TOR but dependent on proteasome-mediated proteolysis.

protein synthesis needed to account for the fast chemotropic responses measured here. We have detected activation of eIF4E and eIF-4EBP1 just 5 min after the addition of Sema3A and netrin-1 and a marked increase in radiolabeled proteins by 10 min. Rough estimates of ribosomal number from immunofluorescent materials suggest that growth cones contain around 500–1000 ribosomes (our unpublished data), some of which are positioned in the fine filopodia. Given that an average protein takes around 2 min to be synthesized (Yewdell, 2001), if all of the ribosomes are active, there is potential for thousands of proteins to be synthesized within minutes of the reception of a guidance stimulus. It is quite possible that only a few copies of a protein are needed in a localized part of the growth cone (perhaps in a single filopodium) to initiate a response. Indeed, localized changes in the growth cone such as the elevation of calcium are sufficient to induce growth cone turning (Zheng, 2000). Our findings lead us to speculate that protein synthesis and/or degradation may occur focally in a sub-region of the growth cone following filopodial contact with a guidance cue. This could potentially cause rapid asymmetric changes in the cytoskeleton necessary for turning.

The direction of the turning responses of growth cones to soluble guidance cues depends on the level of cyclic nucleotides (Ming et al., 1999; Song et al., 1997, 1998). Poo and colleagues (Song et al., 1998; Song and Poo, 1999) have identified two groups of guidance molecules: those whose responses are dependent on the level of cAMP (group 1) and those dependent on the level of cGMP (group 2). Our finding that all the responses to group 1 cues tested (netrin-1, BDNF, and LPA) are inhibited by proteasome inhibitors, while those induced by the group 2 cue, Sema3A, are not, raises the possibility that cAMP-dependent cues require proteasome-mediated proteolysis. Indeed, there is evidence in *Dictyostelium* that the level of cAMP that mediates chemotaxis is controlled in part by degradation of a cAMP-specific phosphodiesterase (Reg A) (Mohanty et al., 2001). It will be necessary to test other group 2 cGMP-dependent guidance cues, such as neurotrophin-3 (NT-3) (Song et al., 1998), to determine whether cGMP-dependent guidance cues as a group do not require proteasome-mediated proteolysis.

Our findings reveal a striking parallel between synaptic plasticity and axon guidance. Functional studies have demonstrated that protein synthesis is required for neurotrophin-induced synaptic plasticity in hippocampal neurons (Kang and Schuman, 1996) and for the establishment of long-term facilitation in *Aplysia* neurons (Martin et al., 1997). Ribosomes are localized beneath postsynaptic sites in adult neurons (Steward and Levy, 1982), and local translation of dendritically localized mRNAs plays a critical role in synaptic plasticity (Martin et al., 2000). Inhibition of protein synthesis in hippocampal neurons blocks LTD within 5 min (Huber et al., 2000), implying a role for rapid dendritically localized protein synthesis. We have shown that rapamycin blocks netrin-1 and Sema3A-stimulated protein synthesis in isolated retinal growth cones. Kandel and colleagues found that rapamycin inhibits long-term facilitation induced by repeated presentations of serotonin at *Aplysia* synapses in culture (Casadio et al., 1999), further suggesting that the mechanisms underlying electrical activity- and guidance cue-induced translation are similar.

A further parallel between axon guidance and synaptic plasticity is in a role for the ubiquitin-proteasome system. Ubiquitin-dependent mechanisms have been implicated in axon guidance (Aguilera et al., 2000; Muralidhar and Thomas, 1993; Oh et al., 1994; Poeck et al., 2001), synaptogenesis (DiAntonio et al., 2001), and synaptic plasticity (Chain et al., 1999; Hegde et al., 1993). In *Aplysia*, Schwartz and colleagues (Hegde et al., 1993) showed that long-term facilitation is regulated by cAMP-stimulated proteasome-mediated proteolysis of the regulatory subunit of PKA. Our findings also suggest a role for cAMP in mediating chemotropic responses via the ubiquitin-proteasome pathway. These parallels between synaptic plasticity and axon guidance probably reflect a requirement in both systems for spatially restricted subcellular reorganization in response to localized stimuli. Other types of localized cellular responses such as directed cell migration may be similarly controlled. Interestingly, chemotaxis in *Dictyostelium* and leukocytes requires PI-3 kinase (Chung et al., 2001), also important for netrin-1-induced chemotropic responses (Ming et al., 1999) and which we have shown is upstream of netrin-1-induced protein synthesis.

The finding that synthesis and degradation are criti-

cally important to growth cone responses to chemotropic cues raises the interesting question of which protein(s) are synthesized and degraded. Candidate proteins are cytoskeletal proteins or those that control cytoskeletal dynamics such as actin binding proteins. We have found that rapamycin completely inhibits netrin-1- and Sema3A-induced protein synthesis (Figure 4T). Since rapamycin, in contrast to anisomycin and cycloheximide, is not a global inhibitor of translation but inhibits translation of 5′ terminal oligopyrimidine (5′TOP) mRNAs, this suggests that mRNAs translated in response to netrin-1 and Sema3A may contain a 5′-TOP sequence adjacent to the N^7-methylguanosine cap structure. β-actin mRNA is a potential candidate translated in response to guidance cues since it is localized to growth cones (Zhang et al., 1999). Disruption of β-actin mRNA and protein localization to the growth cone by antisense oligonucleotides directed against the 3′-untranslated region (UTR) leads to impaired motility of growth cones, suggesting the existence of a novel mechanism to influence growth cone dynamics involving the regulated transport of mRNA (Zhang et al., 2001) and possible local translation. Since the directional response of a growth cone to a guidance cue depends on cyclic nucleotide levels (Song et al., 1998), it is possible that cyclic nucleotides play a role in specifying which proteins are translated. Such specification may involve the transport of specific mRNAs to the growth cone followed by local translation.

Proteins destined for rapid destruction may contain regions enriched in proline, glutamic acid, serine, and threonine known as a PEST sequence (reviewed in Rechsteiner and Rogers, 1996). Candidate degraded proteins in the growth cone, therefore, include those containing PEST sequences. Significantly, components of the cAMP-signaling pathway are known to be regulated by the ubiquitin-proteasome system. For example, the regulatory and catalytic subunits of PKA contain PEST sequences that may be unmasked upon their dissociation facilitating degradation (Rechsteiner, 1990). A role for the degradation of PKA has been shown in long-term facilitation in *Aplysia* (Hegde et al., 1993) and for Reg A, a cAMP-specific phosphodiesterase (Mohanty et al., 2001), that controls cell differentiation in *Dictyostelium*. In addition, the netrin-1 receptor deleted in colorectal cancer (DCC) undergoes ubiquitination and subsequent degradation (Hu et al., 1997) providing another level of control for the netrin-1 signaling pathway by proteasome-mediated proteolysis. Further to its classical role in tagging proteins for destruction by the 26S proteasome, ubiquitin is able to target proteins to the endosomal pathway for degradation by lysosomal proteases (Hicke, 1999, 2001) and plays a role in signal transduction (Deng et al., 2000). Our findings using the proteasome inhibitors LnLL and lactacystin indicate that netrin-1, LPA, and BDNF-induced chemotropic responses require the protein degradative capacity of the proteasome but do not exclude additional roles for ubiquitin in mediating chemotropic responses of growth cones.

Growth cones, like other subcellular compartments, contain protein synthetic and degradative machinery in close proximity, suggesting that protein synthesis and degradation occur close to each other and are highly coordinated, as in other cell types (Yewdell, 2001). It has been estimated that 30% of newly synthesized cellular proteins are rapidly degraded (Schubert et al., 2000) implying a continual cycle of protein synthesis and degradation occurs in the cell. We propose that growth cone steering is regulated by precise control of protein levels both temporally and spatially and that guidance cues in vivo elicit distinct directional responses via differential effects on translation and proteasome-mediated proteolysis.

Experimental Procedures

Retinal Cultures and Collapse Assays

Eye primordia from stage 24, 32, and 35/36 embryos were cultured as described previously (de la Torre et al., 1997). Cultures for collapse assays were grown for 24 hr at room temperature on glass coverslips precoated with 10 μg/ml poly-L-lysine (Sigma) and 10 μg/ml laminin (Sigma). Collapse assays were performed as described (Luo et al., 1993) with the following modifications: 100 μl of COS-7 cell supernatant with or without Sema3A (Ohta et al., 1999; plasmids were gifts from K. Ohta) and 1 μM L-α-Lysophosphatidic acid (LPA) were added to each culture for 10 min. For collapse assays, supernatant containing 0.5 collapsing units (CU) (Luo et al., 1993) of Sema3A was used, which corresponds to approximately 75% retinal growth cone collapse of growth cones from stage 35/36 embryonic retina cultured for 24 hr. Values are presented as percent collapse ± standard error of the mean (SEM) from a minimum of four independent experiments.

Growth Cone Turning Assays

Stable gradients of protein were formed by pulsatile ejection of Sema3A (1.9 CU), control supernatant, or netrin-1 (5 μg/ml; a gift from M. Tessier-Lavigne), or 50 μg/ml brain-derived neurotrophic factor (BDNF, Sigma) or phosphate-buffered saline (PBS) as control (de la Torre et al., 1997; Lohof et al., 1992). For turning assays with netrin-1, eye primordia from stage 24 embryos were cultured for 16–26 hr on coverslips coated with 100 μg/ml poly-L-lysine (Sigma) and 1 μg/ml fibronectin (Sigma) or for 6–12 hr coated with 10 μg/ml poly-L-lysine (Sigma) and 10 μg/ml laminin (Sigma) at room temperature prior to assaying. For turning assays with Sema3A, eye primordia from stage 32 embryos were plated on coverslips coated with 10 μg/ml poly-L-lysine (Sigma) and 5 μg/ml laminin and grown for 16–26 hr at room temperature prior to assaying. The number of growth cones assayed in each condition was between 18 and 36. Statistical analyses were carried out using the StatView software package.

Pharmacological Agents

The following pharmacological agents were bath-applied to cultures immediately prior to the application of Sema3A, netrin-1, BDNF, or LPA in the collapse and turning assays. 40 μM anisomycin (Sigma; inhibits the peptidyltransferase activity on the ribosome), 25 μM cycloheximide (Sigma; inhibits the translocation reaction on ribosomes), 10 μg/ml α-amanitin (Calbiochem; inhibits RNA polymerase II), 50 μM sodium arsenite (Sigma; activates JNK/p38 MAP kinase), 10 nM Rapamycin (Calbiochem; inhibits TOR); 50 nM wortmannin (Biomol; inhibits PI-3 kinase), 10 μM LY294002 (Sigma; inhibits PI-3 kinase). 50 μM N-Acetyl-Leu-Leu-NorLeu-Al (LnLL) (Sigma; a proteasome inhibitor), 10 μM Lactacystin (Calbiochem; a specific inhibitor of the proteasome), 10 μg/ml protease inhibitor cocktail (PIC, leupeptin, chymostatin, pepstatin A and E64 [Sigma], inhibitors of non-proteasome-mediated proteolysis). 100 μM adenosine 3′,5′-cyclic monophosphorothioate, Sp-isomer (SpcAMPS, Calbiochem; membrane-permeable agonist of protein kinase A), 100 μM adenosine 3′,5′-cyclic monophosphorothioate, Rp-isomer (RpcAMPS, Calbiochem; membrane permeable antagonist of protein kinase A), 100 μM guanosine 3′,5′-cyclic monophosphate, 8-Bromo (8-BrcGMP, Calbiochem; membrane-permeable agonist of protein kinase G), 10 μM guanosine, 3′,5′-cyclic monophosphorothioate, 8-(4-Choloro-phenylthio), Rp-isomer (RpcGMPS, Calbiochem; membrane-permeable antagonist of protein kinase G).

Antibodies and Digital Quantitation of Immunofluorescence

Ser65-phosphorylated eIF-4EBP1 was detected in 24 hr stage 35/36 retinal explant cultures using an anti-phospho-4EBP1 (Ser65) anti-

body (Cell Signaling Technology). Ser209 phosphorylated eIF-4E was detected in 24 hr stage 35/36 retinal explant cultures using an anti-*Xenopus* phospho-eIF-4E (Ser209) (Fraser et al., 1999) antibody (a gift from S. Morley). The labeling observed with the antibodies was blocked with their respective phoshopeptides (gifts from Cell Signaling Technology and S. Morley). Ubiquitin conjugated to proteins was detected using the FK2 antibody (Everett, 2000; Fujimuro et al., 1994) (Affiniti Research Products Limited). Cultures were treated with 0.5 CU Sema3A, 300 ng/ml netrin-1, or 1 μM LPA for 5 min, prior to fixation. The immunofluorescence intensities of 35–40 growth cones for each condition from four independent experiments were measured. To obtain quantitative measurements of immunofluorescence, growth cones were randomly selected with phase optics at 100×, and images were captured with a Quantix camera (Photometrics), and the outline was traced digitally using IP Lab Spectrum P Software (Scanalytics Inc). A fluorescent image was then captured, and the amount of fluorescence within the area of the growth cone was calculated digitally. The level of background fluorescence in an adjacent area was similarly calculated and subtracted from the growth cone value to give a final intensity measurement (Campbell et al., 2001; Hopker et al., 1999).

Capped mRNA was detected in retinal growth cones with the H20 antibody (Bochnig et al., 1987), a gift from R. Lührmann. Ribosomes were detected with an anti-ribosomal P0 antibody (Immunovision). Proteasome components were labeled in growth cones using an anti-20S proteasome "core" antibody (Affiniti Research Products Limited) and an anti-*Xenopus* α-4 subunit (Tokumoto et al., 1999), a gift from T. Tokumoto. The signalosome was labeled with an anti-COP9 (csn8) antibody (Affiniti Research Products Limited). Ubiquitin and the ubiquitin-activating enzyme E1were labeled in growth cones with an anti-ubiquitin (Calbiochem) and anti-E1 (Chemicon) antibodies.

Measurement of Protein Synthesis in Isolated Growth Cone Cultures

Protein synthesis in retinal cultures was detected by the incorporation of L-[4,5-^{3}H]-Leucine (Amersham) in trichloroacetic acid (TCA) precipitated proteins using scintillation counting. Given the small amount of growth cone material, it was not feasible to measure the amount of proteins. Therefore, care was taken to size-match samples throughout the experiments. Equal sized pieces of embryonic retinal tissue (stage 35/36) were plated on laminin-coated coverslips (six per coverslip) and cultured for 24 hr. Explant tissue was removed by cutting free from the neurites and the coverslips with approximately the same numbers of severed neurites attached were transferred into leucine-free medium and randomly assigned treatment with inhibitors (anisomycin, cycloheximide, α-amanitin, wortmannin, and rapamycin) for 5 min prior to the addition of L-[4,5-^{3}H]-Leucine (specific activity 138 Ci/mmol). Sema3A, netrin-1, or LPA were added immediately after addition of L-[4,5-^{3}H]-Leucine, and 10 min later cultures were washed in leucine-containing medium (60% L15) (GIBCO, Life Technologies) and fixed with 25% TCA for 30 min. Coverslips with TCA-fixed neurites attached were inserted singly into scintillation vials, and radioactivity was measured with a scintillation counter. Each experiment was done in duplicate and was repeated a minimum of three times. Control coverslips (L-[4,5-^{3}H]-Leucine without guidance molecule) were included with each experimental condition.

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